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| <p>(54) Title: <b>A METHOD FOR ENHANCING AMPLIFICATION IN THE POLYMERASE CHAIN REACTION EMPLOYING PEPTIDE NUCLEIC ACID (PNA)</b><br/>(57) Abstract<br/><p>A process for producing a particular nucleic acid sequence from a given sequence of DNA or RNA in amounts which are large compared to the amount initially present, using PNAs in conjunction with the polymerase chain reaction (PCR) is disclosed.</p></p>  |                  |  |

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A METHOD FOR ENHANCING AMPLIFICATION IN THE  
POLYMERASE CHAIN REACTION EMPLOYING PEPTIDE  
NUCLEIC ACID (PNA)

5

BACKGROUND OF THE INVENTION

The present invention relates to a process for  
amplifying nucleic acid sequences present in a  
sample. More specifically, the present invention  
relates to a method of employing peptide nucleic  
10 acids (PNA) to enhance the amplification of nucleic  
acid sequences via the polymerase chain reaction.  
Still more specifically, the present invention  
relates to a process for producing a particular  
nucleic acid sequence from a given sequence of DNA or  
15 RNA in amounts which are large compared to the amount  
initially present, using PNAs in conjunction with the  
polymerase chain reaction (PCR).

The polymerase chain reaction has, since its  
conception in April 1983, become a standard technique  
20 for amplification of nucleic acid sequences. In this  
process one or more specific nucleic acid sequences  
present in a nucleic acid or mixture thereof are  
amplified using primers and agents for polymerization  
and then detecting the amplified sequence. The  
25 extension product of one primer, when hybridized to  
the other, becomes a template for the production of  
the desired specific nucleic acid sequence, and vice  
versa, and the process is repeated as often as is  
necessary to produce the desired amount of the  
30 sequence.

The DNA or RNA may be single- or double-  
stranded, and may be a relatively pure species or a  
component of a mixture of nucleic acids. The PCR  
process utilizes a repetitive reaction to accomplish  
35 the amplification of the desired nucleic acid  
sequence.

Indeed, in the approximately ten years since its  
discovery, the PCR technique has been widely employed  
in the field of molecular biology in detecting and

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identifying nucleic acid sequences. For example, the polymerase chain reaction (PCR) has been extensively used to amplify DNA loci in genome mapping, linkage studies, genetic diagnostics, forensics, and  
5 paternity testing. The wide-spread use of this technique is testimony to the utility of the process.

Unfortunately, the polymerase chain reaction is not without problems. The PCR process depends upon multiple steps of annealing and melting (separation  
10 of two complementary strands). Under ideal conditions, one can envision the separation of the two complementary strands of double-stranded DNA, annealing of the complementary primers, and elongation of the primers with a polymerase to  
15 produce two molecules of double-stranded DNA. This cycle is repeated while the quantity of the desired sequence grows exponentially.

However, in most contexts in which the polymerase chain reaction is employed, situations are  
20 less than ideal. Often DNA of interest to the biotechnological community contains a number of repeat or near-repeat sequences. Additionally, nucleic acid sequences frequently contain a number of internally self-complementary sequences. These  
25 repeating and self-complementary sequences increase the likelihood that the nucleic acid sequence will experience interstrand and intrastrand interactions, possibly resulting in a nucleic acid strand folding in upon itself.

30 Further, as the polymerase chain reaction progresses, the concentration of complementary DNA sequences increases exponentially, while the concentration of primer commonly remains constant or decreases. This can result in the product of the  
35 interim cycles of the polymerase chain reaction

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successfully competing with the primers required for the next cycle of elongation. Such competition can significantly reduce the efficiency and yield of the polymerase chain reaction.

5       Moreover, diploid DNA poses additional challenges. Robust amplification of allelic sequences is often difficult, particularly when there is a significant size difference between the two alleles. Smaller alleles simply amplify more  
10       efficiently. This phenomenon, known as differential amplification, results in the generation of more copies of the smaller allele and thus a more intense band on the subsequent electrophoresis gel. At best, the larger allele is underrepresented relative to the  
15       smaller allele, at worst, differential amplification can result in allelic dropout, in which the larger allele amplifies so poorly relative to the smaller allele that the larger allele can neither be visualized nor detected. Differential amplification  
20       and allelic dropout can complicate genetic analyses.

      The terms "peptide nucleic acid" and "PNA" refer to a DNA analog with a backbone consisting of N-(2-aminoethyl)glycine units. To this backbone, analogous to DNA, are attached the nucleobases - for  
25       DNA, adenine, guanine, cytidine, and thymine. The individual monomeric units of PNA can be synthesized to furnish a PNA chain having a specific sequence of bases. The synthesis of such PNA chains is detailed in various publications, including Science 254, 1497  
30       (1991); J. Am. Chem. Soc. 114, 9677 (1992); J. Am. Chem. Soc. 114, 1895 (1992); J. Chem. Soc. Chem. Comm. 800 (1993); Proc. Nat. Acad. Sci. USA 90, 1667 (1993); Intercept Ltd. 325 (1992); J. Am. Chem. Soc. 114, 9677 (1992); Nucleic Acids Res. 21, 197 (1993);  
35       J. Chem. Soc. Chem. Commun. 518 (1993); Anti-Cancer

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Drug Design 8, 53 (1993); Nucleic Acids Res. 21, 2103 (1993); Org. Proc. Prep. 25, 457 (1993); CRC Press 363 (1992); J. Chem. Soc. Chem. Commun. 800 (1993); J. Am. Chem. Soc. 115, 6477 (1993); Nature 365, 566 (1993); ABRF News Vol. 4, No. 3 (1993); Science 258, 1481 (1992); WO 8-92/20702; and WO 92/20703, the contents of which are incorporated herein by reference. Additionally, specific sequences of PNA are commercially available from BioSearch Div., PerSeptive Biosystems, Inc., Bedford, MA.

PNA has been demonstrated to be a potent DNA mimic in terms of sequence-specific annealing. Experimental results (discussed in the above publications) have demonstrated that at physiological ionic strength a PNA/DNA duplex is generally 1° C per base pair more stable thermally than the corresponding DNA/DNA duplex. Other experimental data has indicated that PNA is more stable in the cell than DNA; that PNA binds to DNA or RNA 50-100 times more tightly than either DNA or RNA; that PNA can invade and displace double-stranded DNA (dsDNA); and that the backbone of PNA adopts a helical conformation. However, a single base mismatch in a PNA/DNA duplex is much more destabilizing than a mismatch in the corresponding DNA/DNA duplex. Furthermore, PNA does not function as a primer for DNA polymerase.

Recently, PNAs have been used to detect single base mutations through PCR clamping. In Nucleic Acids Research 21, 5332 (1993), PNAs were reported to form a PNA/DNA complex which effectively blocked the formation of a PCR product. However, this PNA-directed PCR clamping results in a blocking of PCR amplification, rather than an enhancement of the polymerase chain reaction.

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Accordingly, there remains a need for an efficient and effective method of amplifying existing nucleic acid sequences present in a sample using the polymerase chain reaction.

5 SUMMARY OF THE INVENTION

Accordingly, an object of the present invention is to provide a method for enhancing amplification of nucleic acid sequences using the polymerase chain reaction.

10 Another object of the present invention is to provide a kit for detecting a nucleic acid sequence of interest.

Still another object of the present invention is to provide a method of cloning into a vector a  
15 nucleic acid sequence of interest.

Still another object of the present invention is to provide an amplified nucleic acid sequence.

Still another object of the present invention is to provide a method for replicating a target nucleic  
20 acid strand with a polymerase to produce a complementary nucleic acid strand.

Still another object of the present invention is to provide a method for replicating a target nucleic acid strand with a thermostable DNA polymerase to  
25 produce a complementary nucleic acid strand.

Still another object of the present invention is to provide a method for replicating a target nucleic acid strand with a polymerase.

These and other objects of the present invention  
30 are achieved by means of a process for detecting the presence or absence of at least one nucleic acid sequence of interest in a sample containing a nucleic acid or a mixture of nucleic acids, comprising treating the sample with at least one PNA oligomer  
35 which binds within the region of nucleic acid to be

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amplified. More specifically, the present invention resides in a process for amplifying one or more specific nucleic acid sequences present in a nucleic acid or mixture thereof using oligonucleotide primers and an agent for polymerization, employing at least one PNA oligomer to reduce the incidence of interstrand and intrastrand interactions, and then isolating and/or detecting the amplified sequence. This method is expected to be more efficient and effective than the conventional PCR technique for producing large amounts of nucleic acid from a target sequence, especially with DNA or RNA that contains one or more repeat or near-repeat sequences.

More specifically, the present invention provides a process for detecting the presence or absence of at least one nucleic acid sequence of interest in a sample containing a nucleic acid or mixture of nucleic acids, wherein the sample is suspected of containing said sequence or sequences, which process comprises the steps of treating the sample with at least one PNA oligomer which binds within the nucleic acid sequence to be amplified; and polymerizing a nucleic acid sequence which is complementary to the nucleic acid sequence of interest.

In another preferred embodiment of the present invention, a method of amplifying a nucleic acid sequence of interest in a sample containing at least one nucleic acid, comprises: (a) treating the sample with (i) an agent for polymerization, (ii) at least one oligonucleotide primer which binds to a strand of each different nucleic acid sequence of interest, (iii) at least one PNA oligomer which is substantially complementary to a portion of and which binds within the nucleic acid sequence of interest at



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a position different from the position at which the oligonucleotide primer binds, and (iv) four different nucleoside triphosphates, forming a first mixture; (b) heating the first mixture to within a first temperature range so as to denature the nucleic acid and to separate any of the at least one oligonucleotide primer and any of the at least one PNA oligomer which is bound to the nucleic acid, forming a second mixture; (c) cooling the second mixture to within a second temperature range at which the at least one PNA oligomer binds to the nucleic acid sequence of interest, forming a third mixture; (d) bringing the third mixture to within a third temperature range at which the at least one oligonucleotide primer binds to the nucleic acid sequence of interest and at which an extension product is synthesized from the oligonucleotide primer on each strand, provided the nucleic acid sequence of interest is present, said synthesis employing the nucleic acid as a template, and whereby the at least one PNA oligomer is displaced from the nucleic acid sequence of interest during said synthesis.

In still another preferred embodiment, the present invention relates to a kit for detecting at least one nucleic acid sequence of interest in a sample, comprising: (a) a container containing at least one oligonucleotide primer for each strand of each nucleic acid sequence of interest, each of said at least one oligonucleotide primer being substantially complementary to a strand of each nucleic acid sequence of interest; (b) a container containing at least one PNA oligomer which is substantially complementary to a portion of and which binds within each nucleic acid sequence of interest

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at a position different from the position at which the at least one oligonucleotide primer binds; and (c) a container containing each of four different nucleoside triphosphates.

- 5 In still another preferred embodiment, the present invention pertains to a process for cloning into a vector a nucleic acid sequence of interest in a sample containing at least one nucleic acid, comprising: (a) treating the sample with (i) an agent  
10 for polymerization, (ii) at least one oligonucleotide primer containing a restriction site at its 5' end and which is substantially complementary to and binds to a strand of each different nucleic acid sequence of interest, (iii) at least one PNA oligomer which is  
15 substantially complementary to a portion of and which binds within the nucleic acid sequence of interest at a position different from the position at which the oligonucleotide primer binds, and (iv) four different nucleoside triphosphates, forming a first mixture;  
20 (b) heating the first mixture to within a first temperature range so as to denature the nucleic acid and to separate any of the at least one oligonucleotide primer and any of the at least one PNA oligomer which is bound to the nucleic acid,  
25 forming a second mixture; (c) cooling the second mixture to within a second temperature range at which the at least one PNA oligomer binds to the nucleic acid, forming a third mixture; (d) bringing the third mixture to within a third temperature range at which  
30 the at least one oligonucleotide primer binds to the nucleic acid sequence of interest and at which an extension product is synthesized from the oligonucleotide primer on each strand, provided the nucleic acid sequence of interest is present, said  
35 synthesis employing the nucleic acid as a template,

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and whereby the at least one PNA oligomer is displaced from the nucleic acid sequence of interest during said synthesis (e) adding to the product of step (d) a restriction enzyme which cleaves at said  
5 restriction site to thereby obtain cleaved products; and (f) ligating the cleaved products into at least one cloning vector.

The steps detailed above may be repeated until the desired level of sequence amplification is  
10 obtained.

In still another preferred embodiment, the present invention pertains to a method for replicating a target nucleic acid strand with a polymerase to produce a complementary nucleic acid  
15 strand, comprising: (A) providing a peptide nucleic acid (PNA) which, (1) is substantially complementary in sequence to nucleobases in at least a portion of the target strand, (2) anneals with said portion of the target strand, and (3) when annealed with said  
20 portion of the target strand, does not serve as a replication initiation site for the polymerase; and (B) annealing the PNA to the target strand prior to initiating replication thereby to enhance replication by the polymerase during production of the  
25 complementary nucleic acid strand.

In still another preferred embodiment, the present invention pertains to a method for replicating a target nucleic acid strand with a thermostable DNA polymerase to produce a  
30 complementary nucleic acid strand, comprising: (A) providing a peptide nucleic acid (PNA) which, (1) is substantially complementary in sequence to nucleobases in at least a portion of the target strand, (2) anneals with said portion of the target  
35 strand, and (3) when annealed with said portion of

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the target strand, does not serve as a replication initiation site for the polymerase; and (B) annealing the PNA to the target strand prior to initiating replication by the polymerase thereby to enhance  
5 replication by the polymerase during production of the complementary nucleic acid strand.

In still another preferred embodiment, the present invention pertains to a method for replicating a target nucleic acid strand with a  
10 polymerase comprising: (A) providing a target nucleic acid strand having a multiplicity of nucleobase sequence repeats; (B) annealing a PNA to the target strand which: (1) is substantially complementary in sequence to at least a portion of one of the repeats;  
15 (2) anneals with the at least a portion of one of the repeats thereby to form a target strand-PNA complex; and (3) when annealed with the at least a portion of one of the repeats, does not serve as a replication initiation site for the polymerase; and (C)  
20 incubating the complex in the presence of a polymerase, such that the polymerase enhances replication of the target strand as compared with when the target strand is not complexed with the PNA.

#### BRIEF DESCRIPTION OF THE DRAWINGS

25 A more complete appreciation of the invention and many of the attendant advantages thereof will be readily obtained as the same become better understood by reference to the following detailed description when considered in connection with the accompanying  
30 drawings, wherein:

Figure 1 provides a schematic illustration of the conventional polymerase chain reaction.

Figure 2 illustrates the chemical structure of the monomeric subunits of PNA and deoxyribonucleic

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acid (DNA), where B is the nucleobase and R<sup>1</sup> is either a hydrogen atom or lysine amide.

Figure 3 shows a naturally occurring deoxyribooligonucleotide oligomer (A) and PNA oligomer (B).

Figure 4 provides a schematic illustration of the PNA-enhanced polymerase chain reaction.

Figure 5 is an autoradiograph illustrating the results of a polymerase chain reaction obtained in the absence of a PNA oligomer and with varying concentrations of a PNA oligomer.

Figure 6 is an autoradiograph illustrating the results of the PNA-enhanced polymerase chain reaction and the conventional PCR run with various samples of DNA.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In an attempt to enhance the amplification of nucleic acid sequences using the polymerase chain reaction, the program of research directed to diminishing the interstrand and intrastrand interactions which decrease the efficiency of the PCR reaction was undertaken. That investigation resulted in the discovery that PNA oligomers, which are substantially complementary to a portion of the nucleic acid sequence of interest but different from that portion of the nucleic acid sequence to which the oligonucleotide primers bind, apparently sufficiently block the nucleic acid functioning as a template, such that the template is unavailable for interactions, either interstrand or intrastrand, while allowing amplification of the sequence to proceed.

The proposed mechanism will become apparent in the following exemplary comparison of the

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conventional polymerase chain reaction and the PNA-enhanced PCR reaction.

In the conventional PCR reaction, as illustrated in Figure 1, a double-stranded DNA molecule, for example, containing the desired sequence comprised of complementary strands is utilized as the nucleic acid. The first and each subsequent reaction cycle extension of each oligonucleotide primer on the original template will produce one new ssDNA molecule product of indefinite length which terminates with only one of the primers. These products, referred to as "long products," will accumulate in a linear fashion; that is, the amount present after any number of cycles will be proportional to the number of cycles.

The long products thus produced will act as templates for one or the other of the oligonucleotide primers and the at least one PNA oligomer during subsequent cycles and will produce molecules of the desired sequence. These molecules will also function as templates for one or the other of the oligonucleotide primers and the at least one PNA oligomer, producing additional amounts of the desired sequence. Thus a chain reaction can be sustained which will result in the accumulation of the desired sequence at an exponential rate relative to the number of cycles.

In the PNA-enhanced PCR reaction, as illustrated in Figure 4, a DNA sequence of interest is placed in solution with two oligonucleotide primers, one oligonucleotide primer for each strand of each nucleic acid sequence of interest, one or more PNA oligomers which bind within the region of nucleic acid to be amplified but in a position different from that which the oligonucleotide primers bind, a

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polymerase, and a mixture of four nucleoside triphosphates, deoxyadenosine triphosphate, deoxythymidine triphosphate, deoxyguanosine triphosphate, and deoxycytidine triphosphate. The mixture is heated to denature the double-stranded nucleic acid. Such denaturing can be achieved thermally, usually at a temperature of 80° to 100° C, preferably 95° ± 5° C. It is not necessary to maintain the mixture at the denaturing temperature for any significant length of time. Indeed, in one embodiment of the present invention, the mixture merely reaches the denaturing temperature range and then it is cooled to the PNA oligomer annealing temperature range. Accordingly, a suitable length of time for maintaining this temperature is between an instantaneous period of time and 10 minutes.

The mixture is cooled to allow the one or more PNA oligomers to anneal. The PNA annealing step can occur at any temperature between the denaturing temperature of the double-stranded DNA and the primer annealing temperature. A suitable temperature for the PNA annealing step is a range of from about 70° to 80°C, preferably 72° to 78°C. It is not necessary to maintain the mixture at the PNA annealing temperature for any significant length of time. Indeed, in one embodiment of the present invention, the mixture merely passes through the PNA annealing temperature range as it is cooled to the primer annealing temperature range. Accordingly, a suitable range of time for maintaining the PNA annealing temperature is from an instantaneous period of time up to ten minutes.

The mixture is then brought to a temperature at which the oligonucleotide primers anneal to the DNA template and an extension product from the primers

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using the nucleic acid sequence as a template is synthesized. A suitable temperature for the primer annealing and primer extension product synthesis step is 40° to 80° C. The mixture is  
5 maintained at the primer annealing and primer extension product synthesis step for a period of time ranging from an instantaneous period of time to 30 minutes.

These steps constitute one cycle of the  
10 PNA-enhanced PCR reaction. To initiate the next cycle, the resultant product of the reaction, two double-stranded DNA molecules, are denatured as in the first step of this cycle.

In a preferred embodiment of the present  
15 invention, the oligonucleotide annealing step and the extension product synthesis step are carried out at two separate temperature ranges. The mixture resulting from annealing of the at least one PNA oligomer is brought to a temperature at which the  
20 oligonucleotide primers anneal to the DNA template. A suitable temperature for the primer annealing step is 40° to 72°C. The mixture is maintained at the primer annealing temperature for a period of time ranging from an instantaneous period of time to 5  
25 minutes.

After the primers have annealed to the DNA templates, the mixture is heated to the primer extension product synthesis temperature. Preferably, this primer extension product synthesis temperature  
30 ranges from 60° - 80° C. The mixture is maintained at the primer extension product synthesis temperature for a period of time ranging from an instantaneous period of time to 10 minutes.

It is postulated that, upon denaturation of the  
35 double-stranded DNA sequence of interest and cooling



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to the PNA annealing temperature, the one or more PNA oligomers bind to the complementary regions of the DNA at a region of the nucleic acid sequence which is different from those regions to which the

5 oligonucleotide primers bind. This binding of the one or more PNA oligomers diminishes or eliminates the annealing of the DNA, either internally with a self-complementary region or externally with a complementary strand of DNA. Upon further cooling to

10 the oligonucleotide primer annealing temperature, the primers bind to the DNA templates. Because the PNA oligomers bind to different sites than those to which the oligonucleotide primers bind, the PNA annealing does not interfere with primer annealing.

15 Upon subsequent warming to the polymerization temperature, the primers are extended by a polymerase. This extension of the primer results in displacement of the one or more PNA oligomers, allowing the synthesis of an extension product

20 through the region to which the one or more PNA oligomers were bound. This displacement of the one or more PNA oligomers during extension of the primer is presumed to result from displacement of the PNA oligomer by the primer extension product, possibly

25 augmented by partial thermal denaturation of the DNA/PNA complex.

The PNAs are synthesized by adaptation of standard peptide synthesis procedures, either in solution or on a solid phase. The synthesis of PNAs

30 is described in Science 254, 1497 (1991); J. Am. Chem. Soc. 114, 9677 (1992); J. Am. Chem. Soc. 114, 1895 (1992); J. Chem. Soc. Chem. Comm. 800 (1993); Proc. Nat. Acad. Sci. USA 90, 1667 (1993); Intercept Ltd. 325 (1992); J. Am. Chem. Soc. 114, 9677 (1992);

35 Nucleic Acids Res. 21, 197 (1993); J. Chem. Soc.

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Chem. Commun. 518 (1993); Anti-Cancer Drug Design 8, 53 (1993); Nucleic Acids Res. 21, 2103 (1993); Org. Proc. Prep. 25, 457 (1993); CRC Press 363 (1992); J. Chem. Soc. Chem. Commun. 800 (1993); J. Am. Chem. Soc. 115, 6477 (1993); Nature 365, 566 (1993); ABRF News Vol. 4, No. 3 (1993); Science 258, 1481 (1992); WO 8-92/20702; and WO 92/20703, the contents of which are incorporated herein by reference.

The present invention comprises a method using PNA to enhance the efficiency of the PCR amplification of nucleic acid sequences. If present during PCR amplification, PNA allows fragments of different size to be more effectively and more evenly amplified. Differential amplification is less apparent and as a result the risk of misclassification is greatly reduced. In addition, fragments of same or similar size are also amplified more effectively.

The one or more PNA oligomers are selected such that blocking of the nucleic acid template occurs without halting the PCR reaction. The PNA molecule must anneal to the DNA template before the complementary DNA strand. However, it is preferred that the PNA be sufficiently destabilized at the primer extension temperature to allow the polymerase to dislodge it and extend through to the end of the template.

The term "PNA oligomer" as used herein is defined as a molecule comprised of two or more PNA monomers. Its exact size will depend on many factors, which in turn depend on the ultimate function or use of the PNA oligomer. In one preferred embodiment of the present invention, the PNA oligomer comprises 5 - 20 PNA monomers, and more preferably 8 - 16 PNA monomer units.

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Additionally, the sequence of the PNA oligomer is designed to be substantially complementary to a nucleic acid sequence. This means that the one or more PNA oligomers must be sufficiently complementary as to anneal with their respective complementary strands. Therefore, the sequence of PNA oligomer need not reflect the exact sequence of the template. Non-complementary bases or longer sequences can be interspersed in the sequence, provided that the sequence has sufficient complementarity with the sequence of the strand to be amplified to hybridize therewith. Within the context of a preferred embodiment, substantial complementarity of a PNA oligomer designates a situation in which there is not more than two mismatches between the nucleobase sequence of the PNA oligomer and the complementary DNA sequence.

The term "oligonucleotide" as used herein in referring to primers is defined as a molecule comprised of two or more deoxyribonucleotides or ribonucleotides, preferably more than three. Its exact size will depend on many factors, which in turn depend on the ultimate function or use of the oligonucleotide.

The term "primer" as used herein refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed in conditions such that synthesis of a primer extension product which is complementary to a nucleic acid strand is induced, i.e., in the presence of nucleotides and an agent for polymerization, such as a DNA polymerase, and at a suitable temperature and pH. The primer is preferably single-stranded for maximum efficiency in

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amplification, but may alternatively be double-stranded. If double-stranded, the primer is first treated to separate its strands before being used to prepare extension products. Preferably, the primer  
5 is an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the agent for polymerization. The exact lengths of the primers will depend on many factors, including temperature  
10 and the source of the primer. For example, depending on the complexity of the target sequence, the oligonucleotide primer typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides. Short primer molecules generally  
15 require cooler temperatures to form sufficiently stable complexes with the template.

The primers are selected to be "substantially" complementary to one or both strands of each specific sequence to be amplified. This means that the  
20 primers must be sufficiently complementary to anneal with their respective strands. Therefore, the sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end  
25 with the remainder of the sequence being complementary to the strand. Alternatively, non-complementary bases or longer sequences can be interspersed in the sequence, provided that the sequence has sufficient complementarity with the  
30 sequence of the strand to be amplified to hybridize therewith and thereby form a template for synthesis of the extension product of the other primer.

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to

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bacterial enzymes each of which cut double-stranded DNA at or near a specific nucleotide sequence.

As used herein, the term "DNA polymorphism" refers to the condition in which two or more  
5 different nucleotide sequences coexist in the same interbreeding population in a DNA sequence.

The present invention is directed to a process for amplifying one or more desired specific nucleic acid sequences suspected of being in a nucleic acid.  
10 Because large amounts of a specific sequence may be produced by this process, the present invention may be used for improving the efficiency of cloning DNA or messenger RNA and for amplifying a target sequence to facilitate detection thereof.

15 In general, the present process involves a chain reaction for producing, in exponential quantities relative to the number of reaction steps involved, at least one specific nucleic acid sequence given that the ends of the required sequence are known in  
20 sufficient detail that oligonucleotide primers can be synthesized which will anneal to the ends, and that at least a portion of the sequence different from the ends to which the primers bind is known in sufficient detail that one or more PNA oligomers can be  
25 synthesized which will anneal to the sequence. The product of the chain reaction will be a discrete nucleic acid duplex with termini corresponding to the ends of the specific primers employed.

Any source of nucleic acid, in purified or  
30 nonpurified form, can be utilized as the starting nucleic acid or acids, provided it is suspected of containing the specific nucleic acid sequence desired. Thus, the process may employ, for example, DNA or RNA, including messenger RNA, which DNA or RNA  
35 may be single-stranded or double-stranded. In

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addition, a DNA-RNA hybrid which contains one strand of each may be utilized. A mixture of any of these nucleic acids may also be employed, or the nucleic acids produced from a previous amplification reaction herein using the same or different primers may be so utilized. The specific nucleic acid sequence to be amplified may be only a fraction of a larger molecule or can be present initially as a discrete molecule, so that the specific sequence constitutes the entire nucleic acid. It is not necessary that the sequence to be amplified be present initially in a pure form; it may be a minor fraction of a complex mixture, such as a portion of the  $\beta$ -globin gene contained in whole human DNA or a portion of nucleic acid sequence due to a particular microorganism which organism might constitute only a very minor fraction of a particular biological sample. The starting nucleic acid may contain more than one desired specific nucleic acid sequence which may be the same or different. Therefore, the present process is useful not only for producing large amounts of one specific nucleic acid sequence, but also for amplifying simultaneously more than one different specific nucleic acid sequence located on the same or different nucleic acid molecules.

The nucleic acid or acids may be obtained from any source, for example, from plasmids such as pBR322, from cloned DNA or RNA, or from natural DNA or RNA from any source, including bacteria, yeast, viruses, and higher organisms, such as plants or animals. DNA or RNA may be extracted from blood or tissue material, such as chorionic villi or amniotic cells, by a variety of techniques such as that described by Maniatis et al., Molecular Cloning: A

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Laboratory Manual, (New York: Cold Spring Harbor Laboratory, 1982), pp. 280-281.

Any specific nucleic acid sequence can be produced by the present process. It is only  
5 necessary that a sufficient number of bases at both ends of the sequence and at some point intermediate to the two ends of the sequence be known in sufficient detail so that two oligonucleotide primers and one or more PNA oligomers can be prepared which  
10 will anneal to the strands of the desired sequence and at relative positions along the sequence such that an extension product synthesized from one primer, when it is separated from its template (complement), can serve as a template for extension  
15 of the other primer into a nucleic acid of defined length. The greater the knowledge about the bases at both ends of the sequence and at the intermediate point, the greater can be the specificity of the primers and the PNA oligomers for the target nucleic  
20 acid sequence, and thus the greater the efficiency of the process.

It will be understood that the word oligonucleotide primer and PNA oligomer as used hereinafter may refer to more than one  
25 oligonucleotide primer or PNA oligomer, particularly in the case where there is some ambiguity in the information regarding the terminal sequences or intermediate sequence of the fragment to be amplified. For instance, in the case where a nucleic  
30 acid sequence is inferred from protein sequence information a collection of primers and oligomers containing sequences representing all possible codon variations based on degeneracy of the genetic code will be used for each strand.

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The oligonucleotide primers may be prepared using any suitable method, such as, for example, the phosphotriester and phosphodiester methods described above, or automated embodiments thereof. In one such automated embodiment, diethylphosphoramidites are used as starting materials and may be synthesized as described by Beaucage et al., Tetrahedron Letters 22, 1859-1862 (1981). One method for synthesizing oligonucleotides on a modified solid support is described in U.S. Pat. No. 4,458,066. It is also possible to use a primer which has been isolated from a biological source, such as a restriction endonuclease digest.

The specific nucleic acid sequence is produced by using the nucleic acid containing that sequence as a template. If the nucleic acid contains two strands, it is necessary to separate the strands of the nucleic acid before it can be used as the template, either as a separate step or simultaneously with the synthesis of the primer extension products. This strand separation can be accomplished by any suitable denaturing method including physical, chemical or enzymatic means. One physical method of separating the strands of the nucleic acid involves heating the nucleic acid until it is completely (> 99%) denatured. Typical heat denaturation may involve temperature ranging from about 80° to 100° C for times ranging from an instantaneous period of time to 10 minutes. Strand separation may also be induced by an enzyme from the class of enzymes known as helicases or the enzyme RecA, which has helicase activity and is known, in the presence of riboATP, to denature DNA. The reaction conditions suitable for separating the strands of nucleic acids with helicases are described by Cold Spring Harbor



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Symposia on Quantitative Biology, Vol. XLIII "DNA: Replication and Recombination" (New York: Cold Spring Harbor Laboratory, 1978), B. Kuhn et al., "DNA Helicases", pp. 63-67, and techniques for using RecA are reviewed in C. Radding, Ann. Rev. Genetics, 16:405-37 (1982).

Preferably, a molar excess, usually about  $10^{14}$ :1 primer:template of the two oligonucleotide primers is added to the buffer containing the separated template strands. It is understood, however, that the amount of complementary strand may not be known if the process herein is used for diagnostic applications, so that the amount of primer relative to the amount of complementary strand cannot be determined with certainty. As a practical matter, however, the amount of primer added will generally be in molar excess over the amount of complementary strand (template) when the sequence to be amplified is contained in a mixture of complicated long-chain nucleic acid strands. A large molar excess is preferred to improve the efficiency of the process.

If the original nucleic acid containing the sequence to be amplified is single-stranded, its complement is synthesized by adding one or two oligonucleotide primers thereto. If an appropriate single primer is added, a primer extension product is synthesized in the presence of the primer, an agent for polymerization and the four nucleotides described below. The product will be partially complementary to the single-stranded nucleic acid and will anneal with the nucleic acid strand to form a duplex of unequal length strands which may then be separated into single strands as described above to produce two single separated complementary strands. Alternatively, two appropriate primers may be added

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to the single-stranded nucleic acid and the reaction carried out.

If the original nucleic acid constitutes the sequence to be amplified, the primer extension products produced will be completely complementary to the strands of the original nucleic acid and will anneal therewith to form a duplex of equal length strands to be separated into single-stranded molecules.

Preferably, a molar excess, usually about  $10^{14}$ :1 primer:template of the PNA oligomer is added to the buffer containing the template. It is understood, however, that the amount of complementary strand may not be known if the process herein is used for diagnostic applications, so that the amount of PNA oligomer relative to the amount of complementary strand cannot be determined with certainty. As a practical matter, however, the amount of PNA oligomer added will generally be in molar excess over the amount of complementary strand (template) when the sequence to be amplified is contained in a mixture of complicated long-chain nucleic acid strands. A large molar excess is preferred to improve the efficiency of the process.

When the complementary strands of the nucleic acid or acids are separated, whether the nucleic acid was originally double or single-stranded, the strands are ready to be used as a template for the synthesis of additional nucleic acid strands. This synthesis can be performed using any suitable method. Generally it occurs in a buffered aqueous solution, preferably at a pH of 7-9, most preferably about 8.

The deoxyribonucleoside triphosphates dATP, dCTP, dGTP and TTP are also added to the synthesis mixture in adequate amounts and the resulting

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solution is heated to about 90° - 100° C. for from an instantaneous period of time to 10 minutes, preferably from 1 to 4 minutes. After this heating period the solution is allowed to cool to from 40° - 80° C, which is preferable for the primer hybridization. To the cooled mixture is added an agent for polymerization, and the reaction is allowed to occur under conditions known in the art. This synthesis reaction may occur at from room temperature up to a temperature above which the agent for polymerization no longer functions efficiently. Thus, for example, if heat-stable DNA polymerase is used as the agent for polymerization, the temperature is generally no greater than about 72° C.

The agent for polymerization may be any compound or system which will function to accomplish the synthesis of primer extension products, including enzymes. Suitable enzymes for this purpose include, for example, Taq polymerase, AMPLITAQ DNA polymerase (available from Perkin Elmer-Cetus), E. coli DNA polymerase I, Klenow fragment of E. coli DNA polymerase I, T4 DNA polymerase, other available DNA polymerases, reverse transcriptase, and other enzymes, including heat-stable enzymes, which will facilitate combination of the nucleotides in the proper manner to form the primer extension products which are complementary to each nucleic acid strand. Generally, the synthesis will be initiated at the 3' end of each primer and proceed in the 5' to 3' direction along the template strand, until synthesis terminates, producing molecules of different lengths. There may be agents, however, which initiate synthesis at the 5' end and proceed in the other direction, using the same process as described above.

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The newly synthesized strand and its complementary nucleic acid strand form a double-stranded molecule which is used in the succeeding steps of the process. In the next step, the strands  
5 of the double-stranded molecule are separated using any of the procedures described above to provide single-stranded molecules.

New nucleic acid is synthesized on the single-stranded molecules. Additional agent for  
10 polymerization, nucleotides, PNA oligomers, and oligonucleotide primers may be added, if necessary, for the reaction to proceed under the conditions prescribed above. Again, the synthesis will be initiated at one end of the oligonucleotide primer  
15 and will proceed along the single strands of the template to produce additional nucleic acid. After this step, half of the extension product will consist of the specific nucleic acid sequence bounded by the two primers.

20 The steps of strand separation, PNA oligomer annealing, oligonucleotide primer annealing, and extension product synthesis can be repeated as often as needed to produce the desired quantity of the desired nucleic acid sequence. The amount of the  
25 nucleic acid sequence produced will accumulate in an exponential fashion.

When it is desired to produce more than one nucleic acid sequence from the first nucleic acid or mixture of nucleic acids, the appropriate number of  
30 different oligonucleotide primers and PNA oligomers are utilized. For example, if two different specific nucleic acid sequences are to be produced, four primers and two or more PNA oligomers are utilized. Two of the primers are specific for one of the  
35 nucleic acid sequences and the other two primers are

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specific for the second nucleic acid sequence. At least one of the PNA oligomers is specific for each nucleic acid sequence. In this manner, each of the two different specific sequences can be produced exponentially by the present process.

The present invention can be performed in a step-wise fashion, where after each step new reagents are added, or simultaneously, where all reagents are added at the initial step, or partially step-wise and partially simultaneous, where fresh reagent is added after a given number of steps. If a method of strand separation, such as heat, is employed which will inactivate the agent for polymerization, as in the case of a heat-labile enzyme, then it is necessary to replenish the agent for polymerization after every strand separation step. The simultaneous method may be utilized when a number of purified components, including an enzymatic means such as helicase, is used for the strand separation step. In the simultaneous procedure, the reaction mixture may contain, in addition to the nucleic acid strand(s) containing the desired sequence, the strand-separating enzyme (e.g., helicase), an appropriate energy source for the strand-separating enzyme, such as ATP, the four nucleotides, the oligonucleotide primers in molar excess, the PNA oligomers in molar excess, and the inducing agent, e.g., Taq polymerase or the Klenow fragment of *E. coli* DNA polymerase I. If heat is used for denaturation in a simultaneous process, a heat-stable inducing agent such as a thermostable polymerase may be employed which will operate at an elevated temperature, preferably 65° - 80° C, depending on the inducing agent, at which temperature the nucleic acid will consist of single and double strands in equilibrium. The upper

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temperature will depend on the temperature at which the enzyme will degrade or the temperature above which an insufficient level of primer hybridization will occur. Each step of the process will occur sequentially, notwithstanding the initial presence of all the reagents. Additional materials may be added as necessary. After the appropriate length of time has passed to produce the desired amount of the specific nucleic acid sequence, the reaction may be halted by inactivating the enzymes in any known manner or by separating the components of the reaction.

The process of the present invention may be conducted continuously. In one embodiment of an automated process, the reaction may be cycled through a denaturing region, a PNA oligomer reagent addition region, an oligonucleotide primer reagent addition region, and a reaction region. In another embodiment, the enzyme used for the synthesis of primer extension products can be immobilized in a column. The other reaction components can be continuously circulated by a pump through the column and a heating coil in series; thus the nucleic acids produced can be repeatedly denatured without inactivating the enzyme.

The steps of this process can be repeated indefinitely, being limited only by the amount of the oligonucleotide primers, the one or more PNA oligomers, the agent for polymerization and nucleotides present. For detection, the number of cycles used is that required to produce a detectable amount, an amount which will depend, e.g., on the nature of the sample. For example, if the sample is pure, fewer cycles may be required than if it is a

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complex mixture. If the sample is human genomic DNA, preferably the number of cycles is from about 10-30.

The amount of original nucleic acid remains constant in the entire process, because it is not replicated. The amount of the long products increases linearly because they are produced only from the original nucleic acid. The amount of the specific sequence increases exponentially. Thus, the specific sequence will become the predominant species.

When a single-stranded nucleic acid is utilized as the template, only one long product is formed per cycle.

The method herein may be utilized to clone a particular nucleic acid sequence for insertion into a suitable expression vector. The vector may then be used to transform an appropriate host organism to produce the gene product of the sequence by standard method of recombinant DNA technology.

The amplification process herein may yield a mixture of nucleic acids, resulting from the original template nucleic acid, the expected target amplified products, and various background non-target products. The amplified product can also be a mixture, if the original template DNA contains multiple target sequences, such as in a heterozygous diploid genome or when there is a family of related genes.

The primers herein may be modified to assist the rapid and specific cloning of the mixture of DNAs produced by the amplification reaction. In such modification the same or different restriction sites are incorporated at the 5' ends of the primers to result in restriction sites at the two ends of the amplified product. When cut with the appropriate enzymes, the amplified product can then be easily

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inserted into plasmid or viral vectors and cloned. This cloning allows the analysis or expression of individual amplified products, not a mixture.

Although the same restriction site can be used  
5 for both primers, the use of different sites allows the insertion of the product into the vector with a specific orientation and suppresses multiple insertions as well as insertions arising from  
amplifications based on only one of the two primers.  
10 The specific orientation is useful when cloning into single strand sequencing vectors, when single strand hybridization probes are used, or when the cloned product is being expressed.

One method to prepare the primers is to choose a  
15 primer sequence which differs minimally from the target sequence. Regions in which each of the primers is to be located are screened for homology to restriction sites appropriate to the desired vector. For example, the target sequence "CAGTATCCGA . . ."  
20 differs by only one base from one containing a BamHI site. A primer sequence is chosen to match the target exactly at its 3' end, and to contain the altered sequence and restriction site near its 5' end (for example, "CAGgATCCGA . . .", where the lower  
25 case letter symbolizes a mismatch with the target sequence). This minimally altered sequence will not interfere with the ability of the primer to hybridize to the original target sequence and to initiate polymerization. After the first amplification cycle  
30 the primer is copied, becomes the target, and matches exactly with new primers. After the amplification process, the products are cleaved with the appropriate restriction enzymes, optionally separated from inhibitors of ligation, such as the nucleoside  
35 triphosphates and salts, by passing over a desalting

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column or molecular weight chromatography column, and inserted by ligation into a cloning vector such as bacteriophage M13. The gene may then be sequenced and/or expressed using well known techniques.

5       The second method for preparing the primers involves taking the 3' end of the primers from the target sequence and adding the desired restriction site(s) to the 5' end of the primer. For the above example, a HindIII site could be added to make the  
10       sequence "cgaagcttCAGTATCCGA . . .", where lower case letters are as described above. The added bases would not contribute to the hybridization in the first cycle of amplification, but would match in subsequent cycles. The final amplified products are  
15       then cut with one or more restriction enzymes and cloned and expressed as described above. The gene being amplified may be, for example, human beta-hemoglobin or the human HLA DQ, DR or DP-alpha and -beta genes.

20       In addition, the process herein can be used for in vitro mutagenesis. The oligodeoxyribonucleotide primers need not be exactly complementary to the DNA sequence which is being amplified. It is only necessary that they be able to hybridize to the  
25       sequence sufficiently well to be extended by the polymerase enzyme or by whatever other inducing agent is employed. The product of a polymerase chain reaction wherein the primers employed are not exactly complementary to the original template will contain  
30       the sequence of the primer rather than the template, thereby introducing an in vitro mutation. In further cycles, this mutation will be amplified with undiminished efficiency because no further mispaired primings are required. The mutant thus produced may  
35       be inserted into an appropriate vector by standard

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molecular biological techniques and might confer mutant properties on this vector such as the potential for production of an altered protein.

5       The process of making an altered DNA sequence as described above could be repeated on the altered DNA using different primers so as to induce further sequence changes. In this way a series of mutated sequences could gradually be produced wherein each new addition to the series could differ from the last  
10       in a minor way, but from the original DNA source sequence in an increasingly major way. In this manner changes could be made ultimately which were not feasible in a single step due to the inability of a very seriously mismatched primer to function.

15       In addition, the primer can contain as part of its sequence a non-complementary sequence, provided that a sufficient amount of the primer contains a sequence which is complementary to the strand to be amplified. For example, a nucleotide sequence which  
20       is not complementary to the template sequence (such as, e.g., a promoter, linker, coding sequence, etc.) may be attached at the 5' end of one or both of the primers, and thereby appended to the product of the amplification process. After the extension primer is  
25       added, sufficient cycles are run to achieve the desired amount of new template containing the non-complementary nucleotide insert. This allows production of large quantities of the combined fragments in a relatively short period of time (e.g.,  
30       two hours or less) using a simple technique.

      If restriction site linkers are incorporated into the primers, then the amplified double-stranded products can be digested with the appropriate restriction enzymes and ligated directly into a  
35       vector for rapid cloning and sequencing. The plaques

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containing the specific amplified target sequences can be identified by hybridizing plaque lift filters with a probe specific for the target sequence.

The method herein may also be used to enable  
5 detection and/or characterization of specific nucleic acid sequences associated with infectious diseases, genetic disorders or cellular disorders such as cancer, e.g., oncogenes. Amplification is useful  
10 when the amount of nucleic acid available for analysis is very small, as, for example, in the prenatal diagnosis of sickle cell anemia using DNA obtained from fetal cells or chorionic villi. Amplification is particularly useful if such an  
15 analysis is to be done on a small sample using non-radioactive detection techniques which may be inherently insensitive, or where radioactive techniques are being employed but where rapid detection is desirable.

For purposes of this invention genetic diseases  
20 may include specific deletions and/or mutations in genomic DNA from any organism, such as, e.g., sickle cell anemia, cystic fibrosis, alpha-thalassemia, beta-thalassemia, and the like. Sickle cell anemia can be readily detected via a RFLP-like analysis  
25 following amplification of the appropriate DNA sequence by the present method. Alpha-thalassemia can be detected by the absence of a sequence, and beta-thalassemia can be detected by the presence of a polymorphic restriction site closely linked to a  
30 mutation which causes the disease.

All of these genetic diseases may be detected by  
amplifying the appropriate sequence and analyzing it. In such a process, for example, a small sample of DNA  
35 from biological material, e.g., amniotic fluid or chorionic villi containing a very low level of the

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desired sequence is amplified, cut with a restriction enzyme, and analyzed.

In another embodiment a small sample of DNA may be amplified to a convenient level and then a further  
5 cycle of extension reactions performed wherein nucleotide derivatives which are readily detectable (such as <sup>32</sup>P-labeled or biotin-labeled nucleoside triphosphates) are incorporated directly into the final DNA product, which may be analyzed by  
10 restriction and electrophoretic separation or any other appropriate method.

In a further embodiment, the nucleic acid may be exposed to a particular restriction endonuclease prior to amplification. Since a sequence which has  
15 been cut cannot be amplified, the appearance of an amplified fragment, despite prior restriction of the DNA sample, implies the absence of a site for the endonuclease within the amplified sequence. The presence or absence of an amplified sequence can be  
20 detected by an appropriate method.

In addition to detecting infectious diseases and pathological abnormalities in the genome of organisms, the process herein can also be used to detect DNA polymorphisms.

25 In a preferred embodiment of the present invention, the nucleic acid sequence which is to be amplified contains at least one sequence portion which is substantially repeated at least once within the nucleic acid sequence. This substantial  
30 repetition encompasses both repetition of identical sequence portions and repetition of nearly-identical sequence portions. Nearly-identical portions are those portions which have 80% or greater sequence homology in a given region.

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In another preferred embodiment of the present invention, the PNA oligomer forms a duplex with a portion of the nucleic acid sequence of interest, which duplex has a melting temperature within the  
5 range of 70° - 80° C, preferably 74° - 78° C. If the nucleic acid sequence of interest contains a sequence portion which is substantially repeated, then, in a preferred embodiment, the PNA oligomer may be a mixture of several PNA oligomers, each of which is  
10 substantially complementary to one or more of the identical or nearly-identical sequence portions.

In still another preferred embodiment of the present invention, the PNA oligomer may be a pair of PNA oligomers, at least a portion of the sequence of  
15 each being complementary to the other member of the pair.

Other features of the invention will become apparent in the course of the following descriptions of exemplary embodiments which are given for  
20 illustration of the invention and are not intended to be limiting thereof.

The PNA H-CCT(G/T)CCGGTGGTC(C/T)TC-NH<sub>2</sub>, a composite of four PNA oligomers, was synthesized and provided by Biosearch Division of PerSeptive  
25 Biosystems, Bedford, MA. Oligonucleotide primers used in the PCR reactions were synthesized by Research Genetics (Huntsville, AL). The sequences of the primers were 5'-GAAACTGGCCTCCAAACACTCCCCGCCG-3' (forward primer), and 5'-  
30 GTCTTGTGGAGATGCACGTGCCCTTGC-3' (reverse primer). The forward primer was end-labeled with T4 polynucleotide kinase and  $\gamma$ -<sup>32</sup>P-ATP.

DNA was extracted from EDTA anticoagulated whole blood using a PureGene DNA Isolation Kit (Gentra  
35 Systems, Minneapolis, MN). Purified DNA from cell

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line K562 was purchased from Promega Corporation (Madison, WI).

Amplification of the D1S80 locus was carried out in a total volume of 12.5 $\mu$ l using 5ng of genomic DNA.

5 Each reaction contained 0.75  $\mu$ M of each primer, 250  $\mu$ M each of dCTP, dATP, dGTP, and TTP, and 1X Stratagene buffer (10mM Tris-HCl [pH 8.8], 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, and 0.01% (w/v) gelatin, and other stabilizers not specified). The PCR reactions were

10 overlaid with 1 drop of mineral oil prior to addition of 0.5 units of Stratagene Taq polymerase (La Jolla, CA), the reaction mixtures were heated to 95° C for 4-10 minutes in a GTC-2 Genetic Thermal Cycler (Precision Scientific, Chicago, IL). Subsequently, 5

15 cycles were carried out each consisting of 95° C for 1.25 minutes for denaturation, 67° C for 30 seconds to allow primer annealing, and 4 minutes at 72° - 76° C for primer extension, followed by 25 cycles each consisting of 95° C for 1.25 minutes, 66° C for 30

20 seconds, and 72° - 76° C for 4 minutes. The final extension was carried out for an additional 5 minutes.

Electrophoresis of the amplified DNA was carried out on 5% Long Ranger denaturing gel (J.T. Baker) at

25 1000 V for 3.5 hr. Following electrophoresis, the polyacrylamide gels were dried using a Savant Slab Gel Dryer (Savant, Inc.). The amplification products were visualized by autoradiography using Kodak XAR5 film and Biotech L-Plus intensifying screens (Fisher

30 Scientific).

Figure 5 is an autoradiograph illustrating the results of the polymerase chain reaction obtained in the absence of PNA oligomers and in the presence of varying amounts of PNA oligomers. Lane 1 represents

35 the reaction detailed above run in the absence of the

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four PNA oligomers. Lanes 2 to 5 represent the above reaction run in the presence of the four PNA oligomers at concentrations of 0.9  $\mu\text{M}$ , 1.2  $\mu\text{M}$ , 1.5  $\mu\text{M}$ , and 1.8  $\mu\text{M}$ , respectively. As is evidenced in the autoradiograph, in the absence of PNA oligomers (Lane. 1), differential amplification does occur. The smaller allele, located at the bottom half of Lane 1 of the autoradiograph, is much more strongly represented relative to the larger allele found in the top half of Lane 1. This differential amplification can result in an erroneous analysis of the sample. In contrast, Lanes 2 to 5, which utilize different concentrations of the PNA oligomers, evidence a much reduced differential amplification.

To evaluate the general application of PNA for enhancing PCR at the D1S80 locus, 4 DNA specimens from routine paternity casework were randomly selected. Each was amplified with PNA (1.5  $\mu\text{M}$ ) and without PNA. All samples were prepared together from the same master mix of reagents and cycled together in the same thermal cycler. Primer extension was carried out at 76°C. The results are shown in figure 6. Lanes 1, 3, 5, and 7 represent polymerase chain reaction run in the presence of PNA, while Lanes 2, 4, 6, and 8 represent experiments run in the absence of PNA. Each DNA specimen run with PNA demonstrated enhanced amplification, particularly of the larger allele.

A PNA can be designed to be shorter than the repeat so as to intentionally create gaps and allow read through by the polymerase. A shortened PNA might be useful for enhancing the amplification of loci consisting of conserved repeats.

The foregoing description of preferred embodiments of the invention has been presented for

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purposes of illustration and description. It is not intended to be exhaustive or to limit the invention to the precise forms disclosed, and obviously many modifications and variations are possible in light of the above teaching. The embodiment was chosen and described in order to best explain the principles of the invention and its practical application to thereby enable others skilled in the art to best utilize the invention in various embodiments and with various modifications as are suited to the particular use contemplated. It is intended that the scope of the invention be defined by the claims appended hereto.



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## WHAT IS CLAIMED IS:

- 1           1. A process for amplifying a nucleic acid  
2           sequence of interest in a sample containing at  
3           least one nucleic acid, comprising the steps of:  
4                 treating the sample with at least one  
5           PNA oligomer which binds within the nucleic acid  
6           sequence to be amplified; and  
7                 polymerizing a nucleic acid sequence  
8           which is complementary to the nucleic acid  
9           sequence of interest.
- 1           2. The process according to claim 1,  
2           further comprising the steps of:  
3                 after said step of treating, annealing  
4           the at least one PNA oligomer to said nucleic acid  
5           sequence of interest;  
6                 subsequently annealing the at least one  
7           oligonucleotide primer to said nucleic acid  
8           sequence of interest; and  
9                 wherein said step of polymerizing  
10          further comprises extending the oligonucleotide  
11          primer so as to synthesize an extension product  
12          employing said nucleic acid sequence of interest  
13          as a template, such that the at least one PNA  
14          oligomer is displaced from the nucleic acid  
15          sequence of interest during synthesis of the  
16          extension product.
- 1           3. The process according to claim 2,  
2           wherein said nucleic acid sequence of interest  
3           includes a sequence portion which is substantially  
4           repeated at least once within the nucleic acid  
5           sequence of interest.
- 1           4. The process according to claim 3,  
2           wherein the at least one PNA oligomer comprises a  
3           mixture of several PNA oligomers, each of which is  
4           substantially complementary to said sequence portion.

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1           5. The process according to claim 2,  
2       wherein said nucleic acid sequence of interest  
3       includes a sequence portion which is repeated at  
4       least once within the nucleic acid sequence of  
5       interest.

1           6. The process according to claim 2,  
2       wherein during the step of annealing the at least  
3       one PNA oligomer, said at least one PNA oligomer  
4       forms a duplex with a portion of the nucleic acid  
5       sequence of interest, said duplex having a melting  
6       temperature within the range of 70° to 80° C.

1           7. The process according to claim 6,  
2       wherein said duplex has a melting temperature  
3       within the range of 74° to 78° C.

1           8. The process according to claim 2,  
2       wherein the at least one PNA oligomer comprises a  
3       pair of PNA oligomers, at least a portion of the  
4       sequence of each of said pair being complementary  
5       to the other.

1           9. The process according to claim 2,  
2       wherein said at least one PNA oligomer comprises a  
3       PNA polymer containing 5 to 20 PNA monomers.

1           10. The process according to claim 9,  
2       wherein said at least one PNA oligomer comprises a  
3       PNA polymer containing 8 to 16 PNA monomers.

1           11. A method of amplifying a nucleic  
2       acid sequence of interest in a sample containing  
3       at least one nucleic acid, comprising:

- 4           (a) treating the sample with  
5               i) an agent for polymerization,  
6               ii) at least one oligonucleotide  
7               primer which binds to a strand  
8               of each different nucleic acid  
9               sequence of interest,

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- 10                   iii) at least one PNA oligomer  
11                   which is substantially  
12                   complementary to a portion of  
13                   and which binds within the  
14                   nucleic acid sequence of  
15                   interest at a position  
16                   different from the position at  
17                   which the oligonucleotide  
18                   primer binds, and  
19                   iv) four different nucleoside  
20                   triphosphates, forming a first  
21                   mixture;  
22                   (b) heating the first mixture to within  
23                   a first temperature range so as to denature the  
24                   nucleic acid and to separate any of the at least  
25                   one oligonucleotide primer and any of the at least  
26                   one PNA oligomer which is bound to the nucleic  
27                   acid, forming a second mixture;  
28                   (c) cooling the second mixture to  
29                   within a second temperature range at which the at  
30                   least one PNA oligomer binds to the nucleic acid  
31                   sequence of interest, forming a third mixture;  
32                   (d) bringing the third mixture to  
33                   within a third temperature range at which the at  
34                   least one oligonucleotide primer binds to the  
35                   nucleic acid sequence of interest and at which an  
36                   extension product is synthesized from the  
37                   oligonucleotide primer on each strand, provided  
38                   the nucleic acid sequence of interest is present,  
39                   said synthesis employing the nucleic acid as a  
40                   template, and whereby the at least one PNA  
41                   oligomer is displaced from the nucleic acid  
42                   sequence of interest during said synthesis.

1           12. The method according to claim 11,  
2           comprising repeating steps (b), (c), and (d) at  
3           least one time.

1           13. The method according to claim 11,  
2           further comprising the step of  
3           (e) treating the product of step (d)  
4           under denaturing conditions to separate the  
5           extension product from said template.

1           14. The method according to claim 13,  
2           further comprising the step of  
3           (f) detecting the presence of any of  
4           said nucleic acid sequence of interest.

1           15. The method according to claim 11,  
2           wherein the nucleic acid sequence of interest  
3           includes a sequence portion which is substantially  
4           repeated at least once within the nucleic acid  
5           sequence of interest.

1           16. The method according to claim 15,  
2           wherein the at least one PNA oligomer comprises a  
3           mixture of several PNA oligomers, each of which is  
4           substantially complementary to said sequence  
5           portion.

1           17. The method according to claim 11,  
2           wherein said nucleic acid sequence of interest  
3           includes a sequence portion which is repeated at  
4           least once within the nucleic acid sequence of  
5           interest.

1           18. The method according to claim 11,  
2           wherein during step (c) the at least one PNA  
3           oligomer forms a duplex with a portion of the  
4           nucleic acid sequence of interest, said duplex  
5           having a melting temperature within the range of  
6           70° to 80° C.

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1           19. The method according to claim 18,  
2           wherein said duplex has a melting temperature  
3           within the range of 74° to 78° C.

1           20. The method according to claim 11,  
2           wherein the at least one PNA oligomer comprises a  
3           pair of PNA oligomers, at least a portion of the  
4           sequence of each of said pair being complementary  
5           to the other.

          21. The method according to claim 11,  
          wherein the first temperature range is a range of  
          80° to 100° C.

1           22. The method according to claim 11,  
2           wherein the second temperature range is a range of  
3           70° to 80° C.

1           23. The method according to claim 22,  
2           wherein the second temperature range is a range of  
3           72° to 78° C.

1           24. The method according to claim 11,  
2           wherein the third temperature range is a range of  
3           40° to 80° C.

1           25. The method according to claim 11,  
2           wherein said at least one PNA oligomer comprises a  
3           PNA polymer containing 5 to 20 PNA monomers.

          26. The method according to claim 25,  
          wherein said at least one PNA oligomer comprises a  
          PNA polymer containing 8 to 16 PNA monomers.

1           27. The method according to claim 11,  
2           wherein, while performing steps (c) and (d), the  
3           temperature is continuously varied.

1           28. The method according to claim 11,  
2           wherein, during each of steps (c) and (d), the  
3           temperature is maintained at a substantially  
4           constant value for an interval of time.

1           29. The method according to claim 11,  
2           wherein step (d) comprises the steps of:

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3 (d1) bringing the third mixture to  
4 within a fourth temperature range which is within  
5 said third temperature range and at which the at  
6 least one oligonucleotide primer binds to the  
7 nucleic acid sequence of interest, forming a  
8 fourth mixture;

9 (d2) heating the fourth mixture to  
10 within a fifth temperature range which is within  
11 said third temperature range and at which said  
12 extension product is synthesized from the  
13 oligonucleotide primer on each strand, whereby the  
14 at least one PNA oligomer is displaced from the  
15 nucleic acid sequence of interest, forming a fifth  
16 mixture.

1 30. The method according to claim 29,  
2 comprising repeating steps (b), (c), and (d) at  
3 least one time.

1 31. The method according to claim 29,  
2 further comprising the step of  
3 (e) treating the product of step (d)  
4 under denaturing conditions to separate the  
5 extension product from said template.

1 32. The method according to claim 31,  
2 further comprising the step of

3 (f) detecting the presence of any of  
4 said nucleic acid sequence of interest.

1 33. The method according to claim 29,  
2 wherein the first temperature range is a range of  
3 80° to 100° C.

1 34. The method according to claim 29,  
2 wherein the second temperature range is a range of  
3 70° to 80° C.

1 35. The method according to claim 34,  
2 wherein the second temperature range is a range of  
3 72° to 78° C.

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1           36. The method according to claim 29,  
2           wherein the fourth temperature range is a range of  
3           40° to 72° C.

1           37. The method according to claim 29,  
2           wherein the fifth temperature range is a range of  
3           60° to 80° C.

1           38. A kit for detecting at least one nucleic  
2           acid sequence of interest in a sample, comprising:

3               (a) a container containing at least one  
4               oligonucleotide primer for each strand of each  
5               nucleic acid sequence of interest, each of said at  
6               least one oligonucleotide primer being  
7               substantially complementary to a strand of each  
8               nucleic acid sequence of interest;

9               (b) a container containing at least one  
10              PNA oligomer which is substantially complementary  
11              to a portion of and which binds within each  
12              nucleic acid sequence of interest at a position  
13              different from the position at which the at least  
14              one oligonucleotide primer binds; and

15              (c) a container containing each of four  
16              different nucleoside triphosphates.

1           39. The kit according to claim 38, further  
2           comprising:

3               (d) a container containing an agent for  
4               polymerization.

1           40. The kit according to claim 38 wherein  
2           said nucleic acid of interest includes a sequence  
3           portion which is substantially repeated at least  
4           once within the nucleic acid sequence of interest.

1           41. The kit according to claim 40, wherein  
2           the at least one PNA oligomer comprises a mixture  
3           of several PNA oligomers, each of which is  
4           substantially complementary to said sequence  
5           portion.

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1           42. The kit according to claim 38 wherein  
2       said nucleic acid sequence of interest includes a  
3       sequence portion which is repeated at least once  
4       within the nucleic acid sequence of interest.

1           43. The kit according to claim 38, wherein  
2       the at least one PNA oligomer forms a duplex with  
3       a portion of the nucleic acid sequence of  
4       interest, said duplex having a melting temperature  
5       within the range of 70° to 80° C.

1           44. The kit according to claim 43, wherein  
2       said duplex has a melting temperature within the  
3       range of 74° to 78° C.

1           45. The kit according to claim 38, wherein  
2       the at least one PNA oligomer comprises a pair of  
3       PNA oligomers, at least a portion of the sequence  
4       of each of said pair being complementary to the  
5       other.

1           46. The kit according to claim 38, wherein  
2       said at least one PNA oligomer comprises a PNA  
3       polymer containing 5 to 20 PNA monomers.

1           47. The kit according to claim 46, wherein  
2       said at least one PNA oligomer comprises a PNA  
3       polymer containing 8 to 16 PNA monomers.

1           48. A process for cloning into a vector a  
2       nucleic acid sequence of interest in a sample  
3       containing at least one nucleic acid, comprising:

- 4           (a) treating the sample with  
5               i) an agent for polymerization,  
6               ii) at least one oligonucleotide  
7                     primer containing a  
8                     restriction site at its 5' end  
9                     and which is substantially  
10                    complementary to and binds to  
11                    a strand of each different



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12 nucleic acid sequence of  
13 interest,  
14 iii) at least one PNA oligomer  
15 which is substantially  
16 complementary to a portion of  
17 and which binds within the  
18 nucleic acid sequence of  
19 interest at a position  
20 different from the position at  
21 which the oligonucleotide  
22 primer binds, and  
23 iv) four different nucleoside  
24 triphosphates, forming a first  
25 mixture;  
26 (b) heating the first mixture to within  
27 a first temperature range so as to denature the  
28 nucleic acid and to separate any of the at least  
29 one oligonucleotide primer and any of the at least  
30 one PNA oligomer which is bound to the nucleic  
31 acid, forming a second mixture;  
32 (c) cooling the second mixture to  
33 within a second temperature range at which the at  
34 least one PNA oligomer binds to the nucleic acid,  
35 forming a third mixture;  
36 (d) bringing the third mixture to  
37 within a third temperature range at which the at  
38 least one oligonucleotide primer binds to the  
39 nucleic acid sequence of interest and at which an  
40 extension product is synthesized from the  
41 oligonucleotide primer on each strand, provided  
42 the nucleic acid sequence of interest is present,  
43 said synthesis employing the nucleic acid as a  
44 template, and whereby the at least one PNA  
45 oligomer is displaced from the nucleic acid  
46 sequence of interest during said synthesis

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47 (e) adding to the product of step (d) a  
48 restriction enzyme which cleaves at said  
49 restriction site to thereby obtain cleaved  
50 products; and

51 (f) ligating the cleaved products into  
52 at least one cloning vector.

1 49. The process according to claim 48,  
2 comprising repeating steps (b), (c), and (d) at  
3 least one time.

1 50. The process according to claim 48,  
2 wherein said nucleic acid sequence of interest  
3 includes a sequence portion which is substantially  
4 repeated at least once within the nucleic acid  
5 sequence of interest.

1 51. The process according to claim 50,  
2 wherein the at least one PNA oligomer comprises a  
3 mixture of several PNA oligomers, each of which is  
4 substantially complementary to said sequence  
5 portion.

1 52. The process according to claim 48,  
2 wherein said nucleic acid sequence of interest  
3 includes a sequence portion which is repeated at  
4 least once within the nucleic acid sequence of  
5 interest.

1 53. The process according to claim 48,  
2 wherein during step (c) the at least one PNA  
3 oligomer forms a duplex with a portion of the  
4 nucleic acid sequence of interest, said duplex  
5 having a melting temperature within the range of  
6 70° to 80° C.

1 54. The process according to claim 53,  
2 wherein said duplex has a melting temperature  
3 within the range of 74° to 78° C.

1 55. The process according to claim 48,  
2 wherein the at least one PNA oligomer comprises a

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3 pair of PNA oligomers, at least a portion of the  
4 sequence of each of said pair being complementary  
5 to the other.

1 56. The process according to claim 48,  
2 wherein the first temperature range is a range of  
3 80° to 100° C.

1 57. The process according to claim 48,  
2 wherein the second temperature range is a range of  
3 70° to 80° C.

1 58. The process according to claim 57,  
2 wherein the second temperature range is a range of  
3 72° to 78° C.

1 59. The process according to claim 48,  
2 wherein the third temperature range is a range of  
3 40° to 80° C.

1 60. The process according to claim 48,  
2 wherein said at least one PNA oligomer comprises a  
3 PNA polymer containing 5 to 20 PNA monomers.

1 61. The process according to claim 60,  
2 wherein said at least one PNA oligomer comprises a  
3 PNA polymer containing 8 to 16 PNA monomers.

1 62. The process according to claim 48,  
2 wherein, while performing steps (c) and (d), the  
3 temperature is continuously varied.

1 63. The process according to claim 48,  
2 wherein, during each of steps (c) and (d), the  
3 temperature is maintained at a substantially  
4 constant value for an interval of time.

1 64. The process according to claim 48,  
2 wherein step (d) comprises the steps of:

3 (d1) bringing the third mixture to  
4 within a fourth temperature range which is within  
5 said third temperature range and at which the at  
6 least one oligonucleotide primer binds to the

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7 nucleic acid sequence of interest, forming a  
8 fourth mixture;  
9 (d2) heating the fourth mixture to  
10 within a fifth temperature range which is within  
11 said third temperature range and at which said  
12 extension product is synthesized from the  
13 oligonucleotide primer on each strand, whereby at  
14 least one PNA oligomer is displaced from the  
15 nucleic acid sequence of interest, forming a fifth  
16 mixture.

1 65. The process according to claim 64,  
2 wherein the first temperature range is a range of  
3 80° to 100° C.

1 66. The process according to claim 64,  
2 wherein the second temperature range is a range of  
3 70° to 80° C.

1 67. The process according to claim 66,  
2 wherein the second temperature range is a range of  
3 72° to 78° C.

1 68. The process according to claim 64,  
2 wherein the fourth temperature range is a range of  
3 40° to 72° C.

1 69. The process according to claim 64,  
2 wherein the fifth temperature range is a range of  
3 60° to 80° C.

1 70. An amplified nucleic acid sequence of  
2 interest produced by a process comprising the  
3 steps of:

- 4 (a) treating the sample with  
5 i) an agent for polymerization,  
6 ii) at least one oligonucleotide  
7 primer which binds to a strand  
8 of each nucleic acid sequence  
9 of interest,

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- 10                   iii) at least one PNA oligomer  
11                   which is substantially  
12                   complementary to a portion of  
13                   and which binds within the  
14                   nucleic acid sequence of  
15                   interest at a position  
16                   different from the position at  
17                   which the at least one  
18                   oligonucleotide primer binds,  
19                   and  
20                   iv) four different nucleoside  
21                   triphosphates, forming a first  
22                   mixture;  
23                   (b) heating the first mixture to within  
24                   a first temperature range so as to denature the  
25                   nucleic acid and to separate any of the at least  
26                   one oligonucleotide primer and any of the at least  
27                   one PNA oligomer which is bound to the nucleic  
28                   acid, forming a second mixture;  
29                   (c) cooling the second mixture to within  
30                   a second temperature range at which the at least  
31                   one PNA oligomer binds to the nucleic acid  
32                   sequence of interest, forming a third mixture;  
33                   (d) bringing the third mixture to  
34                   within a third temperature range at which the at  
35                   least one oligonucleotide primer binds to the  
36                   nucleic acid sequence of interest and at which an  
37                   extension product is synthesized from the  
38                   oligonucleotide primer on each strand, provided  
39                   the nucleic acid sequence of interest is present,  
40                   said synthesis employing the nucleic acid as a  
41                   template, and whereby the at least one PNA  
42                   oligomer is displaced from the nucleic acid  
43                   sequence of interest during said synthesis.

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1           71. The amplified nucleic acid sequence  
2           according to claim 70, comprising repeating steps  
3           (b), (c), and (d) at least one time.

1           72. The amplified nucleic acid sequence  
2           according to claim 70, further comprising the step  
3           of           (e) treating the product of step (d)  
4           under denaturing conditions to separate the  
5           extension product from said template.

1           73. The amplified nucleic acid sequence  
2           according to claim 72, further comprising the step  
3           of           (f) detecting the nucleic acid sequence  
4           of interest.

1           74. The amplified nucleic acid sequence  
2           according to claim 70, wherein said nucleic acid  
3           sequence of interest includes a sequence portion  
4           which is substantially repeated at least once  
5           within the nucleic acid sequence of interest.

1           75. The amplified nucleic acid sequence  
2           according to claim 74, wherein the at least one  
3           PNA oligomer comprises a mixture of several PNA  
4           oligomers, each of which is substantially  
5           complementary to said sequence portion.

1           76. The amplified nucleic acid sequence  
2           according to claim 70, wherein said nucleic acid  
3           sequence of interest includes a sequence portion  
4           which is repeated at least once within the nucleic  
5           acid sequence of interest.

1           77. The amplified nucleic acid sequence  
2           according to claim 70, wherein during step (c) the  
3           at least one PNA oligomer forms a duplex with a  
4           portion of the nucleic acid sequence of interest,  
5           said duplex having a melting temperature within  
6           the range of 70° to 80° C.

1           78. The amplified nucleic acid sequence  
2           according to claim 77, wherein said duplex has a

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3 melting temperature within the range of 74° to 78°  
4 C.

1 79. The amplified nucleic acid sequence  
2 according to claim 70, wherein the at least one  
3 PNA oligomer comprises a pair of PNA oligomers, at  
4 least a portion of the sequence of each of said  
5 pair being complementary to the other.

1 80. The amplified nucleic acid sequence  
2 according to claim 70, wherein the first  
3 temperature range is a range of 80° to 100° C.

1 81. The amplified nucleic acid sequence  
2 according to claim 70, wherein the second  
3 temperature range is a range of 70° to 80° C.

1 82. The amplified nucleic acid sequence  
2 according to claim 81, wherein the second  
3 temperature range is a range of 72° to 78° C.

1 83. The amplified nucleic acid sequence  
2 according to claim 70, wherein the third  
3 temperature range is a range of 40° to 80° C.

1 84. The amplified nucleic acid sequence  
2 according to claim 70, wherein said at least one  
3 PNA oligomer comprises a PNA polymer containing 5  
4 to 20 PNA monomers.

1 85. The amplified nucleic acid sequence  
2 according to claim 84, wherein said at least one  
3 PNA oligomer comprises a PNA polymer containing 8  
4 to 16 PNA monomers.

1 86. The amplified nucleic acid sequence  
2 according to claim 70, wherein, while performing  
3 steps (c) and (d), the temperature is continuously  
4 varied.

1 87. The amplified nucleic acid sequence  
2 according to claim 70, wherein, wherein, during  
3 each of steps (c) and (d), the temperature is

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4 maintained at a substantially constant value for  
5 an interval of time.

1 88. The amplified nucleic acid sequence  
2 according to claim 70, wherein step (d) comprises  
3 the steps of:

4 (d1) bringing the third mixture to  
5 within a fourth temperature range which is within  
6 said third temperature range and at which the at  
7 least one oligonucleotide primer binds to the  
8 nucleic acid sequence of interest, forming a  
9 fourth mixture;

10 (d2) heating the fourth mixture to  
11 within a fifth temperature range which is within  
12 said third temperature range and at which said  
13 extension product is synthesized from the  
14 oligonucleotide primer on each strand, whereby the  
15 at least one PNA oligomer is displaced from the  
16 nucleic acid sequence of interest, forming a fifth  
17 mixture.

1 89. The method according to claim 88,  
2 comprising repeating steps (b), (c), and (d) at  
3 least one time.

1 90. The method according to claim 88,  
2 further comprising the step of

3 (e) treating the product of step (d)  
4 under denaturing conditions to separate the  
5 extension product from said template.

1 91. The method according to claim 89,  
2 further comprising the step of

3 (f) detecting the presence of any of  
4 said nucleic acid sequence of interest.

1 92. The amplified nucleic acid sequence  
2 according to claim 88, wherein the first  
3 temperature range is a range of 80° to 100° C.



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1           93. The amplified nucleic acid sequence  
2           according to claim 88, wherein the second  
3           temperature range is a range of 70° to 80° C.

1           94. The amplified nucleic acid sequence  
2           according to claim 93, wherein the second  
3           temperature range is a range of 72° to 78° C.

1           95. The amplified nucleic acid sequence  
2           according to claim 88, wherein the fourth  
3           temperature range is a range of 40° to 72° C.

1           96. The amplified nucleic acid sequence  
2           according to claim 88, wherein the fifth  
3           temperature range is a range of 60° to 80° C.

1           97. A method for replicating a target  
2           nucleic acid strand with a polymerase to produce a  
3           complementary nucleic acid strand, comprising:

- 4                   (A) providing a peptide nucleic acid  
5                   (PNA) which,  
6                   (1) is substantially complementary  
7                   in sequence to nucleobases in  
8                   at least a portion of the  
9                   target strand,  
10                  (2) anneals with said portion of  
11                  the target strand, and  
12                  (3) when annealed with said  
13                  portion of the target strand,  
14                  does not serve as a  
15                  replication initiation site  
16                  for the polymerase; and  
17                  (B) annealing the PNA to the target  
18                  strand prior to initiating  
19                  replication thereby to enhance  
20                  replication by the polymerase  
21                  during production of the  
22                  complementary nucleic acid strand.

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1           98. The method according to claim 97,  
2       wherein step (A) comprises providing a mixture of  
3       PNAs, wherein the nucleobase sequence of at least  
4       one of the PNAs in the mixture is complementary in  
5       sequence to said portion of the target strand.

1           99. The method according to claim 97,  
2       wherein the PNA provided in step (A) comprises 5  
3       to 20 nucleobases complementary in sequence to  
4       said portion of the target strand.

1           100. The method according to claim 99,  
2       wherein the PNA provided in step (A) comprises 8  
3       to 16 nucleobases complementary in sequence to  
4       said portion of the target strand.

1           101. The method according to claim 97,  
2       wherein the target strand comprises a multiplicity  
3       of nucleobase sequence repeats.

1           102. The method according to claim 97,  
2       wherein the polymerase is a DNA polymerase.

1           103. The method according to claim 102,  
2       wherein the DNA polymerase is a thermostable DNA  
3       polymerase.

1           104. The method according to claim 102,  
2       further comprising the step of annealing an  
3       oligonucleotide primer to a second nucleobase  
4       sequence of the target strand thereby to produce a  
5       replication initiation site for the polymerase.

1           105. The method according to claim 103,  
2       further comprising the step of annealing an  
3       oligonucleotide primer to a second nucleobase  
4       sequence of the target strand thereby to produce a  
5       replication initiation site for the polymerase.

1           106. A method for replicating a target  
2       nucleic acid strand with a thermostable DNA  
3       polymerase to produce a complementary nucleic acid  
4       strand, comprising:

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- 5 (A) providing a peptide nucleic acid  
6 (PNA) which,  
7 (1) is substantially complementary  
8 in sequence to nucleobases in  
9 at least a portion of the  
10 target strand,  
11 (2) anneals with said portion of  
12 the target strand, and  
13 (3) when annealed with said  
14 portion of the target strand,  
15 does not serve as a  
16 replication initiation site  
17 for the polymerase; and  
18 (B) annealing the PNA to the target  
19 strand prior to initiating  
20 replication by the polymerase  
21 thereby to enhance replication by  
22 the polymerase during production of  
23 the complementary nucleic acid  
24 strand.

1 107. The method according to claim 106,  
2 wherein step (A) comprises providing a mixture of  
3 PNAs, wherein the nucleobase sequence of at least  
4 one of the PNAs in the mixture is complementary in  
5 sequence to said portion of the target strand.

1 108. The method according to claim 106,  
2 wherein the PNA provided in step (A) comprises 5  
3 to 20 nucleobases complementary in sequence to  
4 said portion of the target strand.

1 109. The method according to claim 108,  
2 wherein the PNA provided in step (A) comprises 8  
3 to 16 nucleobases complementary in sequence to  
4 said portion of the target strand.

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1           110. The method according to claim 106,  
2           wherein the target strand comprises a multiplicity  
3           of nucleobase sequence repeats.

1           111. The method according to claim 106,  
2           further comprising the step of:

3           (C) annealing an oligonucleotide primer  
4           to a second nucleobase sequence of the target  
5           strand thereby to produce a replication initiation  
6           site for the polymerase.

1           112. A method for replicating a target  
2           nucleic acid strand with a polymerase comprising:

3           (A) providing a target nucleic acid  
4           strand having a multiplicity of  
5           nucleobase sequence repeats;

6           (B) annealing a PNA to the target  
7           strand which:

8           (1) is substantially complementary  
9           in sequence to at least a  
10           portion of one of the repeats;

11           (2) anneals with the at least a  
12           portion of one of the repeats  
13           thereby to form a target  
14           strand-PNA complex; and

15           (3) when annealed with the at  
16           least a portion of one of the  
17           repeats, does not serve as a  
18           replication initiation site  
19           for the polymerase; and

20           (C) incubating the complex in the  
21           presence of a polymerase, such that  
22           the polymerase enhances replication  
23           of the target strand as compared  
24           with when the target strand is not  
25           complexed with the PNA.

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1           113. The method according to claim 112,  
2       wherein step (3) comprises providing a mixture of  
3       PNAs, wherein the nucleobase sequence of at least  
4       one of the PNAs in the mixture is complementary in  
5       sequence to said at least a portion of one of the  
6       repeats.

1           114. The method according to claim 112,  
2       wherein the PNA provided in step (B) comprises 5  
3       to 20 nucleobases complementary in sequence to  
4       said at least a portion of one of the repeats.

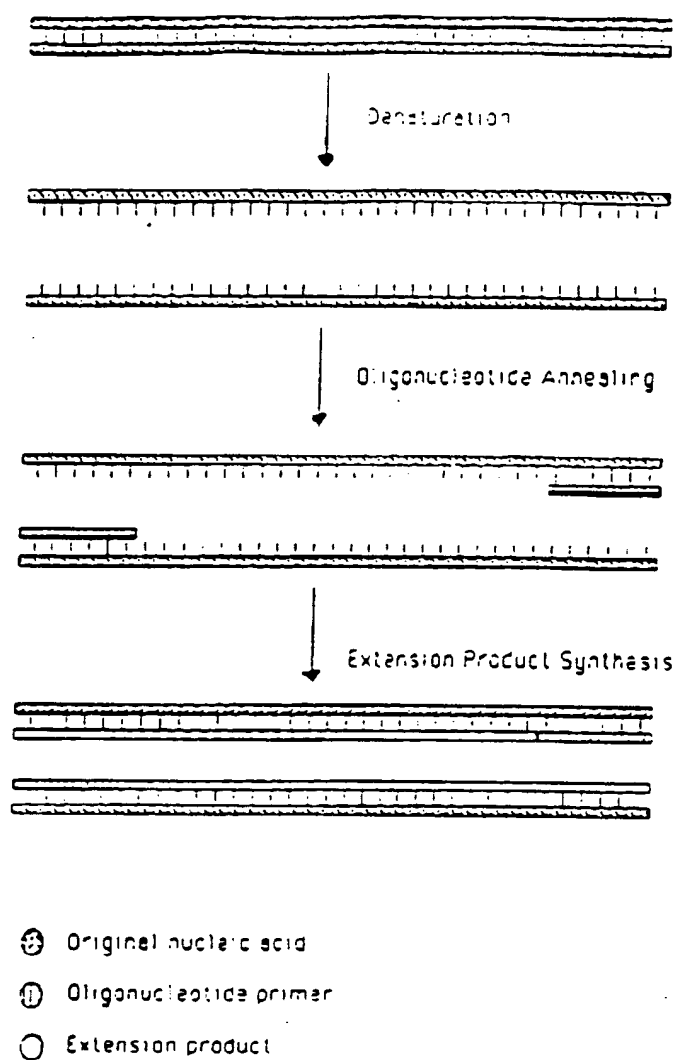
1           115. The method according to claim 114,  
2       wherein the PNA provided in step (B) comprises 8  
3       to 16 nucleobases complementary in sequence to  
4       said at least a portion of one of the repeats.

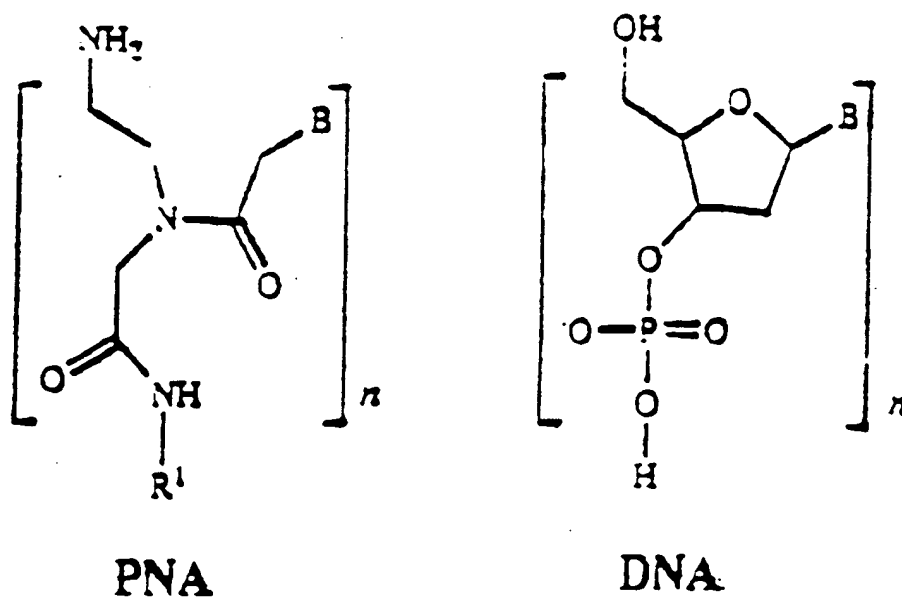
1           116. The method according to claim 112,  
2       wherein the polymerase is a DNA polymerase.

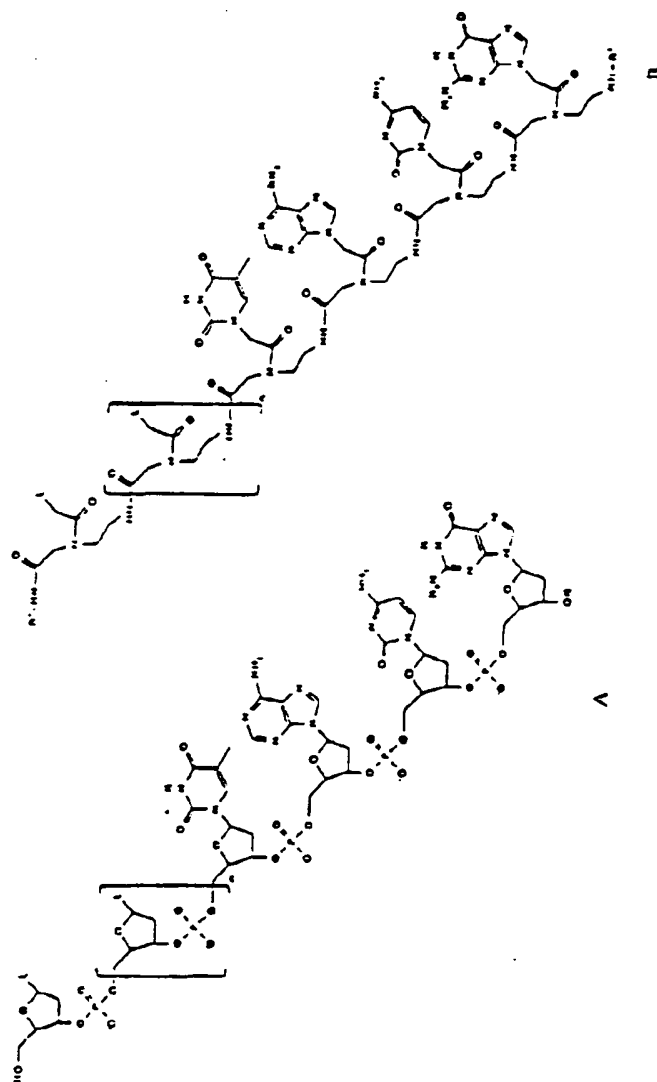
1           117. The method according to claim 116,  
2       wherein the DNA polymerase is a thermostable DNA  
3       polymerase.

1           118. The method according to claim 112  
2       further comprising the step of:

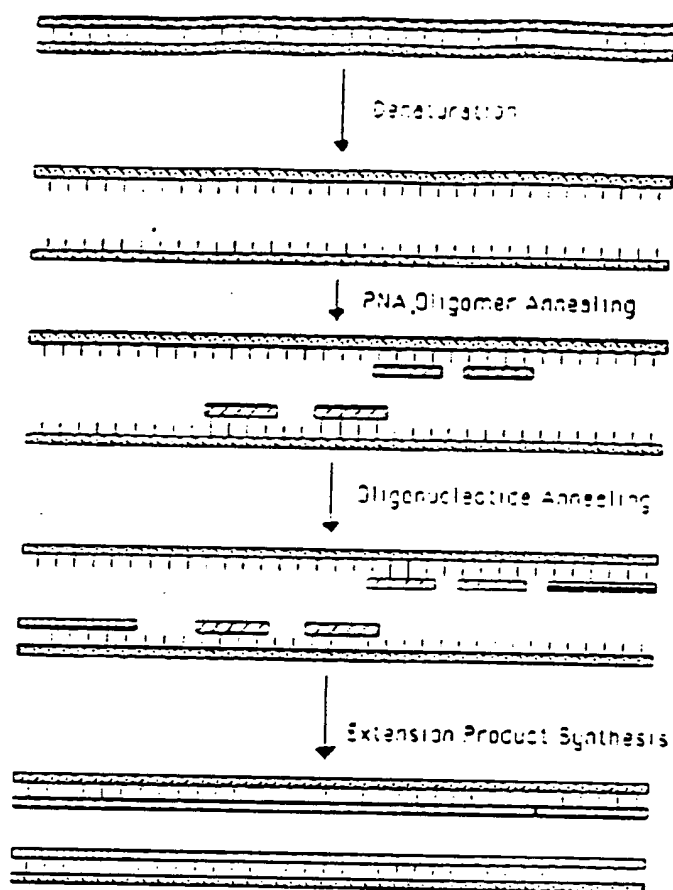
3           (D) annealing a primer to a second  
4               sequence of the target strand  
5               thereby to produce a replication  
6               initiation site for the polymerase.

**Figure 1.**

**Figure 2**

**Figure 3**





- ⊗ Original nucleic acid
- ▢ Oligonucleotide primer
- Extension product
- ▨ PNA Oligomer

**Figure 4**



**Figure 5**



**Figure 6**

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/13345

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : C12Q 1/68; 1/70; C12P 19/34; C07H 21/04; A61K 48/00

US CL : 435/6, 5, 91.2; 536/24.5, 44

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 5, 91.2; 536/24.5, 44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No. |
|-----------|---|-----------------------|
| X         | Nucleic Acids Research, Volume 21, Number 23, issued 02 June 1993, H. Orum et al, "Single base pair mutation analysis by PNA directed PCR clamping", pages 5332-5336, especially pages 5332-5332. | 38-47                 |
| X         | Innis et al, "PCR Protocols, A Guide to Methods and Applications" published 1990 by Academic Press (N.Y.), pages 13-20, see Figure 1.   | 70-88, 92-96          |
| X, P      | WO, A, 94/28171 (PNA DIAGNOSTICS) 08 December 1994, especially page 27.   | 38-47                 |
| P, A      | WO, A, 95/08556 (AMERSHAM INTERNATIONAL PLC) 30 March 1995, especially pages 3-7.   | 1-118                 |

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent (family) annex.

|  |      |  |
|--|------|--|
| * Special categories of cited documents:   | * T  | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention  |
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| * E* earlier document published on or after the international filing date  | * Y* | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is considered with one or more other such documents, such combination being obvious to a person skilled in the art |
| * L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | * A* | document member of the same patent family  |
| * O* documents referring to an oral disclosure, use, exhibition or other means   |      |  |
| * P* document published prior to the international filing date but later than the priority date claimed  |      |  |

Date of the actual completion of the international search

04 JANUARY 1996

Date of mailing of the international search report

01 FEB 1996

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US95/13345

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No. |
|-----------|---|-----------------------|
| A         | Proceedings of the National Academy of Science, Volume 90, issued March 1993, D.Y. Cherny et al, "DNA unwinding upon strand-displacement binding of a thymine-substituted polyamide to double-stranded DNA", pages 1667-1670, especially page 1670. | 1-118                 |

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/13345

## B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, PROMT, JAPIO, BIOBUSINESS, CABA, WPIDS, BIOTECHDS, BIOSIS, EMBASE, MEDLINE, DISSABS, SCISEARCH, AIDSLINE, CANCERLIT, TOXLIT, TOXLINE, USPATFULL

search terms: PNA, peptide nucleic acids, PCR, amplification, helix unwinding, helix destabilization, DNA melting, repeats, hairpins, or palindromes, loops, cloning using PNAs.